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(71) Applicants *(for all designated States except US)*: **AMERSHAM PHARMACIA BIOTECH AB** [SE/SE]; Bjorkgatan 30, S-751 84 Uppsala (SE). **Q-ONE BIOTECH LTD** [GB/GB]; Todd Campus, West Scotland Science Park, Glasgow G20 0XA (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **STADLER, Joachim** [DE/DE]; Schlierbergstrasse 106, 79100 Freiburg (DE). **FAHRENMARK, Johan** [SE/SE]; Engelbrektsvag 48, S-191 92 Sollentuna (SE). **LAGERLUND, Inger** [SE/SE]; Arkitektvagen 56, S-168 32 Bromma (SE).

(74) Agents: **ROLLINS, Anthony, John** et al.; Nycomed Amersham plc, Amersham Laboratories, White Lion Road, Amersham, Buckinghamshire HP7 9LL (GB).

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(54) Title: METHOD FOR PURIFYING PLASMID DNA BY ANION-EXCHANGE CHROMATOGRAPHY

(57) Abstract: A method for purifying plasmid on a bed composed of an anion exchange matrix bed comprising a plurality of anion exchange groups firmly attached to a hydrophilic base matrix. The method is characterized that A. essentially all of the anion exchanging ligands are weak anion exchange ligands, and B. the method comprises the steps: (a) providing a plasmid sample derived from a cleared lysate of recombinant host cells producing the plasmid; (b) contacting said sample with the bed of the anion exchange matrix, said contacting being under conditions enabling binding of said plasmid to said anion exchange matrix; (c) desorbing selectively said plasmid from said anion exchange matrix, and (d) if so desired, regenerating the matrix after an optional cleaning step, and repeating step (b) with another batch of sample provided according to step (a).

METHOD FOR PURIFYING PLASMID DNA BY ANION-EXCHANGE CHROMATOGRAPHY

Technical field.

The present invention relates to purification of plasmids, in particular for the production of pharmaceutical grade plasmids. These processes typically comprise (a) sample preparation, (b) a capture step in which the plasmid is adsorbed to a bed of an anion exchange matrix and subsequently desorbed and collected therefrom, and (c) one or more additional steps for further purifying the desorbed plasmid.

Background technology.

Typically this kind of processes comprises culturing host cells producing the plasmid of interest and lysis of the cells. After precipitation of proteins and RNAs, cell debris together with precipitates are removed from the lysate, for instance by centrifugation. Further contaminants (including additional RNAs) may be removed in subsequent steps. This may be accomplished by additional precipitations/centrifugations and/or size exclusion filtrations including size exclusion chromatography and the like.

Finally the protein and RNA depleted lysate (plasmid sample) is subjected to a capture step on a bed of an anion exchange matrix under conditions giving as high selectivity for the plasmid as possible. The plasmid collected from the capture step may then be subjected to further steps, so called purification and/or polishing steps, in which remaining contaminants are removed, for instance any residual amounts of RNA, proteins and genomic DNA.

WO 9916869 describes a plasmid purfication method focusing on precipitating RNA by the use of a soluble alkali earth metal salt and a subsequent capture step involving anion exchange. Suggested anion exchangers vary from weak to strong anion exchangers (primary, secondary, tertiary and quaternary ammonium groups as anion exchange ligands). The anion exchanging groups are in the preferred variants linked to the base matrices via extenders or tentacles. The elution protocol

is relatively cumbersome, typically starting from a relatively acidic pH for adsorption and then switching gradually to an alkaline pH in order to separately elute RNAs and plasmid DNA. During the pH gradient the ionic strength (conductivity) is changed and gives step-wise desorption of RNA and plasmid DNA. In order to accomplish pharmaceutical grade qualities it is suggested that a polishing anion exchange step should be added after the capture step.

An alternative method is given by Ollivier and Stadler, (Gene Therapy of Cancer (editors Walden et al), Plenum Press, New York (1998) 487-492).

A significant part of the anion exchanging capacity in the most common N,N-dialkyl aminoalkyl anion exchangers derives from quaternary ammonium structures created during the manufacturing process (Lagerlund et al., J. Chromatog, A 796 (1998) 129-140). These structures will remain charged at high pHs, which in turn means disadvantages when cleaning the matrices at increased pHs before reusing them for additional batches of plasmid.

Anion exchangers carrying primary, secondary and tertiary ammonium groups for the isolation of certain compounds having nucleic acid structure or polypeptide structure have been disclosed in WO 9729825 (Amersham Pharmacia Biotech AB). The synthetic methods used avoid creation of quaternary ammonium groups.

Objectives of the present invention.

Production processes for plasmids should be simple and have as few steps as possible. The productivity must be high and give plasmid of acceptable purity in order to make the process cost effective. The processes as such should also be robust and stable and result in yields and/or purities that are not sensitive minor variations in process conditions, for instance in the feedstream. In particular the methods should result in a minimal conversion of covalently closed circle plasmids (CCC) or so-called supercoiled plasmids to relaxed open circle forms (OC).

A pharmaceutical grade plasmid preparation encompasses a plasmid preparation that is approved to be used as a therapeutic in vivo. At present time this considered to mean: supercoiled plasmid > 95 % of the nucleic acid content (w/w), RNA < 2 %, preferably < 1 %, of the nucleic acid content (w/w), chromosomal DNA < 1 % of the plasmid content, endotoxins < 40 EU/mg plasmid, protein content 1 µg/mg DNA, preferably of proteins heterologous to the individual to be treated.

Typically the overall yield of plasmid for the capture step should be > 90 % such as > 95 % of plasmid in the sample to be applied. The ratio CCC/OC plus linearised shouldn't decrease more than 1-5% and is in most cases not efffected by the capture step.

The most important objective of the invention is to provide a method that enables a higher productivity and/or results in a higher purity of the plasmid in fewer and/or simpler steps compared to previously known methods, in particular compared to WO 9916869 (Amersham Pharmacia Biotech AB). One of the objectives is therefore to provide an improved process that gives a plasmid preparation that complies with the figures for one, two, three or more of the contaminants given in the preceding paragraph.

An additional objective is to facilitate recycling of the matrix used in a first process cycle to one or more subsequent process cycles.

A further objective is a facilitated desorption after adsorption in the capture step.

The invention

It has now been discovered that the above-mentioned objectives can be at least partly complied with by a method comprising a capture step in which the anion exchanger used is a weak anion exchanger in which certain weak anion exchanging groups are linked to a hydrophilic base matrix via spacers. Further features that assist in complying with the objectives are given below.

Thus the invention is a method for obtaining a purified plasmid by the use of a bed composed of an anion exchange matrix which has a plurality of anion exchange ligands firmly attached to a hydrophilic base matrix. A characteristic feature is that

A. essentially all of the anion exchanging ligands are weak anion exchange ligands, and

B. the method comprises the steps:

(a) providing a plasmid sample derived from a cleared lysate of host cells producing the plasmid;

(b) contacting the plasmid sample with the anion exchange matrix bed, said contacting being under conditions enabling adsorption of the plasmid to said anion exchange matrix;

(c) desorbing the plasmid from said anion exchange matrix; and

(d) if so desired, regenerating the anion exchange matrix, possibly after cleaning, and repeating step (b) with another batch of sample provided according to step (a).

Steps b-c and step d, if present, are preferably carried out in one and the same vessel, such as a chromatographic column or the like.

Weak anion exchanging groups are protonated nitrogen-containing groups that can be deprotonated in aqueous liquids at moderate pH-values. Illustrative examples are primary, secondary or tertiary ammonium groups, i.e. protonated amine groups, that have a pKa in the range of 5-11.

In preferred modes the matrix has extenders providing for binding between one or more spacers and the matrix.

Typical anion exchange matrices are previously known in the field and are discussed further down under the heading "Matrices, Ligands and Spacers".

The inventive process may also comprise one or more subsequent steps for further purifying the plasmid desorbed in step (c). Such steps may involve adsorption/desorption of the plasmid or of contaminants to an anion exchanger, a reverse

phase matrix, a HIC matrix (hydrophobic interaction chromatography) etc. Extra purification steps may also utilize size exclusion chromatography. There may also adsorption steps before the capture step. Extra purification steps are mostly required for preparing pharmaceutical grades of plasmids. The final one or two purification steps are often called polishing while purification steps between the capture step (step (b) above) and polishing are often called intermediate purification.

The conductivity values and pH values discussed below refer to values measured in water solutions without addition of a water-miscible organic solvent such as ethanol. In case water-miscible solvents are included then conductivity and pH values giving rise to the equivalent effects shall be used. pH and conductivity gradients refer to changes caused by varying the concentration of acids/bases and dissociable salts, respectively.

Preparation of samples to be applied to the anion exchanger (step a).

The plasmid used in the present invention can be of any origin. It may be produced in microorganisms like E. coli and other cells of prokaryotic or eukaryotic origin. Typical host cells are recombinant in the sense that the desired plasmid is not native for them. Host cells harbouring the plasmid can be cultivated in a number of ways, well known in the field, e.g. in an incubator, a bioreactor, a fermentor etc. The plasmid can be of any size. It can have a high copy number or a low copy number per cell. The plasmid can carry any gene (either genomic or synthetic) encoding one or more proteins/polypeptides from a wide variety of sources. Typically the plasmid is a recombinant plasmid. The culturing of host cells as well as the use of the plasmid, e.g. in gene therapy, are well known in the field.

After culturing, the host cells with the plasmid may be washed, for instance by microfiltration on 0.8 µm membranes, to remove media components, and endotoxins, genomic DNA and other

substances released during fermentation. The host cells are collected by centrifugation or filtration, for instance. The cells can be stored, for instance in a freezer, or processed immediately.

In the next step, the cells are lysed according to standard methods. Most commonly used are alkaline lysis or alkaline extraction. See Maniatis et al., (1982) Molecular cloning: A laboratory Manual, Cold Spring Harbour Laboratory Manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, N.Y.; and Birnboim et al., Nucleic Acid Research 7 (1979) 1513-, respectively. Typical lysis methods comprise breaking up the cell walls with an ionic detergent in alkaline milieu, for instance NaOH, and typically in the presence of a chelator. A number of alternative lysis methods have been suggested. WO 9636706 (Merck), for instance, suggest combining host cells, a nonionic detergent, RNase, lysozyme and heating to 70-100°C, for instance in a heat exchanger

Typical detergents for the lysis step are selected amongst

- anionic, for instance sodium dodecyl sulphate and the like, and
- nonionic for instance containing polyethylene and polypropylene oxide monomeric units at one end and an hydrophobic at another end (for instance Triton X100).

Other potential detergent alternatives are zwitterionic detergents. The use of cationic detergents can not be excluded.

Each particular lysis method has their optimal selection of reagents, including also the detergent used.

Typical chelators are able to complex with alkali earth metal ions and other divalent metal ions. Illustrative examples are EDTA and other chelators that have an acceptable toxicity in humans. EGTA [$(HOOCCH_2)_2NCH_2CH_2OCH_2CH_2OCH_2CH_2N(CH_2COOH)_2$] is one candidate. When selecting chelator it may often be beneficial to select the chelator having the best selectivity for the most abundant divalent metal ion in the sample. This means the highest selectivity for calcium in case precipitation with calcium ions has been carried out.

Proteins, RNAs and cell debris, such as membranes in conjunction with parts of chromosomal DNA, are precipitated after cell lysis by adding a protein and/or RNA precipitating water-soluble agent and decreasing the pH to about 5-6. Typical precipitating agent are water-soluble salts, such as potassium acetate. The precipitating agent is preferably added as a concentrated aqueous solutions to a final concentration of above 0.5 M in the lysate. The recommended final concentration is from around 0.58 M with an upper limit of about 1.5 M such as about 1 M. The precipitate formed together with cell debris and the like may be removed by centrifugation and/or filtration to give a cleared lysate. Additional precipitation of RNAs may be obtained by the addition of a water soluble alkali earth metal salt, such as $CaCl_2$, preferably in form of an aqueous solution of about 1-4 M to a final concentration of about 0.1-0.5, preferably about 0.2 M in the cleared lysate. The formed precipitate may be removed in the same way as in the previous step, for instance by filtration through a 0.45 µm filter. Precipitation with an alkali earth metal salt may also be carried out in conjunction with the first precipitating step, i.e. at pH 5-6 in the presence of an ionic detergent.

There are a number of alternative methods for removing proteins and nucleic acids before obtaining a sample suitable for a capture step on an anion exchanger according to the invention. These methods may wholly or partly replace the above-mentioned steps or may be used as complements to further remove undesired nucleic acids, proteins and other contaminants including for instance endotoxins and other bacterial proteins. Thus the crude or the cleared lysate may be treated with organic solvents and/or enzymatically, for instance with RNase. The cleared lysate may also be subjected to size exclusion filtration, for instance size exclusion chromatography or ultrafiltration, in order to decrease the ionic strength (conductivity) and levels of contaminants of lower molecular weight.

The selection of ultrafiltration membrane is often a balance between a high productivity, a high recovery, a sufficient filtration effect and an acceptable low conversion of CCC to OC plasmids. Suitable ultrafiltration membranes typically have a cut-off within the interval of 10,000 - 300,000 KD (apparent molecular size) with preference for 75,000-150,000 KD for 2-6 kbp plasmids. One kind of suitable membranes is made of polyether sulphone which may be provided by Vivascience, Lincoln UK or Filtron/Pall, Northborough, MA, U.S.A.

The sample must have a conductivity (ionic strength) that will allow adsorption to the anion exchanger. Therefore it is normal practice to condition the sample, for instance by dilution, prior to step (b). Conditioning may also comprise pH-adjustment. See below.

Adsorption (step b, capture step)

The conditions during the adsorption step are selected so that binding can take place via ion exchange, i.e.

- pH is selected such that (a) at least a fraction of the ligands is positively charged and (b) the nucleic acids are negatively charged. This typically means a pH value within the interval 3-12 with preference for 4.8-8 and a recommended value being around 5.1.
- The conductivity is below the maximal conductivity permitting binding of the plasmid to the anion exchanger under the conditions applied. This suggests that the conductivity is below the elution conductivity. The conductivity should be within 40-99% of the elution conductivity with optimal values typically being > 80 % with preference for > 90 %, such as 85-95%.

The relatively high conductivity during sample application will facilitate later desorption and increase selectivity between RNA and plasmid.

The term "elution conductivity" refers to the conductivity at which a plasmid bound to an anion exchanger is released from the anion exchanger. Elution conductivity will vary between

different plasmids, anion exchangers and other conditions, for instance pH, temperature etc.

Appropriate values for pH and conductivity during step (b) will depend on factors given in the preceding paragraph and also on kind and amount of contaminating species in the sample etc. This means that all values within the ranges given are not equally applicable in each particular case.

The anion exchanger and the sample to be applied to the anion exchange matrix should have pH and conductivity as close to each other as possible. This means that the anion exchanger prior to step (b) is equilibrated with a buffer providing the desired pH and conductivity.

In many variants of the invention it is beneficial to adjust the concentrations of chelators and detergents in the sample before subjecting the sample to step (b). The important matter then is to adjust their levels to be functionally constant from one batch to another for one and the same process. A practical way of doing this is to add a sufficient excess amount to render fluctuations from the various batches insignificant. It is believed that the presence of a detergent and/or a chelator in the sample to be applied in the capture step will facilitate removal of endotoxins.

The detergent level in the sample is typically > 0.005 % (w/w) such as > 0.01 % (w/w). The upper limit is typically mostly < 0.2 % (w/w) such as < 0.1 % (w/w). Independent of these limits it is believed that there may be advantages in going above the critical micell-forming concentration (CMC) for the detergent under the conditions applied. The detergent may be selected among the same detergents as can be used in the lysis step, but the selected one does not necessarily need to be the same.

The chelator should preferably be in excess for chelating essentially all akaline earth metal ions and other divalent metal ions, which are present in the sample. This typically means chelator concentrations that are ≥ 1 mM such as ≥ 2 mM and ≤ 20 mM such as ≤ 10 mM.

For both anionic detergents and anionic chelators it is important not to include concentration levels that may be deleterious to the capture step.

The protein, RNA and endotoxin content of the plasmid sample to be applied in the capture step should be as low as possible in order to simplify the capture step and minimise the demands for subsequent intermediate purification/polishing steps. Typically the protein content is ≦ 30 mg/ml and the RNA content is ≤ 25 mg/ml. The endotoxin content may be > 200 EU per ml, typically > 40 000 EU per ml. Relative to total nucleic acid content, the plasmid should be present in quantities ≥ 3 % (w/w). Depending on the objective for a particular purification process, the levels may be significantly lower.

Desorption (step c)

Before the next step (step c; desorption) the bed of anion exchange matrix may, if so found necessary, be washed with excess equilibration buffer or any other buffer not interfering with the binding or desorption processes.

Desorption of bound material is accomplished by changing the conditions provided by the eluting buffer. In the inventive process there are three main alternatives

(1) to increase the conductivity,
(2) to increase the pH, and
(3) to combine (1) with (2).

An increase in conductivity is accomplished by increasing the salt concentration of the eluting liquid. Typical salts shall be highly soluble in water are illustrated by salts between halide ions and alkali metals, such as LiCl and NaCl. It is better to use a physiologically acceptable salt if the plasmid is to be used therapeutically. The change can be carried out as a gradient that may be step-wise (one or more steps) or continuous. A continuous or a multistep gradient for increasing conductivity may facilitate separate desorption of negatively charged contaminants that have an elution conductivity between

the starting conductivity and the elution conductivity for the plasmid.

An increase in pH will enable decharging of weak anion exchanging groups and thus a decrease in the interactions between negatively charged compounds (such as plasmids) and the anion exchange matrix. An increase in pH will also decrease the elution conductivity for any negatively charged substance that is bound to the matrix. Thus a pH-gradient may also be used to separately desorb contaminants.

Conductivity and pH gradients can be combined. A conductivity gradient, for instance, can precede a pH-gradient or vice versa. pH-changes and conductivity changes can also be applied in parallel.

Depending on the contaminants concerned, the resolution in the adsorption/desorption step can be enhanced by multistep or continuous gradients. However, this will also complicate the process meaning that single step changes are to be preferred in large-scale processes, if possible.

By applying the principles discussed herein we have managed with the following elution pattern:

(a) the major part of the RNAs in the void or wash;

(b) a very minor part of RNAs in the initiating part of the conductivity gradient; and

(c) the plasmid eluted as a well separated and distinct peak after the initiating part of the gradient.

To accomplish this, adsorption was allowed to take place at a pH where the weak anion exchange groups are at least partially charged and the conductivity is about 5-20% below the elution conductivity. Desorption has taken place at the same pH as the adsorption with an increasing conductivity gradient. This indicates that it might be possible to desorb with a single step conductivity gradient (ionic gradient).

Depending on the contaminants bound in step (b), the anion exchange ligand used etc, other desorbing agents can be used in combination with the above-mentioned three desorbing variants. Examples are non-polar solvents miscible with aqueous liquids, hydrogen-bond-breaking agents (see for instance WO 9729825

(Amersham Pharmacia Biotech AB)) soluble ligand structure analogues etc.

Cleaning step and regeneration step and a second cycle starting from step b (step (d).

After the desorption step the matrix may be regenerated with a regenerating liquid and used in a subsequent cycle of the process for another batch of the plasmid. In cyclic processes there is typically inserted a cleaning step between the desorption and the regeneration step after a certain number of cycles. It is practical to carry out cleaning in the same vessel as used in step (b) and step (c) (cleaning in place).

The number of cycles between two cleaning steps will depend on contaminants in the sample and thus also on the sample preparation and the demand on the final product.

The regeneration liquid may be a solution containing a salt concentration that is the same or higher than in the liquid used for desorption. Typically the salt concentration is ≥ 0.1 M such as ≥ 0.4 M or even ≥ 0.75 M. The pH should be such as to keep the anion exchange ligand charged. The regeneration liquid may be essentially the same as the buffer that is to be used for equilibrating the anion exchanger prior to step (b)

The cleaning liquid may be a solution containing a salt in high concentration and/or an alkaline agent that renders the liquid strongly alkaline. Typical salts are selected among the same salts as for the regeneration liquid. Typical alkaline agents are soluble metal hydroxides, in particular of the group 1A elements, such as NaOH. Preferred concentrations of the alkaline agent and/or salt are from 0.1 M and upwards, such as ≥ 0.4 M or even ≥ 0.75 M. Cleaning liquids devoid of alkaline agents may have the same composition as a regeneration liquid.

The flow velocity and the bed height may sometimes be of importance in order to have a high productivity and hihj dynamic capacity during adsorption/desorption (steps b and c). Typical bed heights for large scale processes are found in the

interval 5 cm to 1 m with preference for the interval 5-30 cm, such as 10-30 cm, in the present invention. Typical flow velocities for the matrices of today are within the interval 10-2000 cm/h with preference for 30 cm/h up to 700 cm/h for packed beds. For expanded beds the same range applies but it can easily be forecasted that even higher flow rates can and will be applied, for instance up to 3000 cm/h. See for instance WO 9717132 (Amersham Pharmacia Biotech AB, Uppsala, Sweden). For large molecules such as DNAs it is often preferred run the processes in the lower half of these velocity ranges. In order to obtain an optimal adsorption and separation it can be advantageous to adapt the flow velocity and the bed height to each other so that the residence time is within the interval 1-30 minutes for each part of the sample. The preferred range is 3-15 minutes. The optimal residence time will depend on porosity, bead size, adsorption strength, concentrations of plasmids etc.

The various steps involved (equilibration, adsorption, desorption, cleaning, regeneration) are preferably carried out in one and the same vessel.

Matrices, ligands and spacers.

The anion exchanging matrix complies with the formula I:

L-----SP-M

in which

- ----- represents that SP is a spacer covalently binding to the ligand L by replacing one group in L.
- M is the base matrix and in the preferred variants comprises so-called extenders (= tentacles). In the matrix M there are further groups L-----SP.
- SP represents a spacer that attaches the ligand L to the base matrix.
- L is the ligand and is selected among weak anion exchange groups.

The base matrix may be any of the traditional base matrices used in the field of liquid chromatography and other adsorption techniques.

The base matrix is hence preferably hydrophilic and in the form of a polymer, which is insoluble and more or less swellable in water. Hydrophobic polymers that have been derivatized to become hydrophilic are included in this definition. Suitable polymers are polyhydroxy polymers, e.g. based on polysaccharides, such as agarose, dextran, cellulose, starch, pullulan, etc. and completely synthetic polymers, such as polyacrylic amide, polymethacrylic amide, poly(hydroxyalkylvinyl ethers), poly(hydroxyalkylacrylates) and polymethacrylates (e.g. polyglycidylmethacrylate), polyvinylalcohols and polymers based on styrenes and divinylbenzenes, and copolymers in which two or more of the monomers corresponding to the above-mentioned polymers are included. Polymers, which are soluble in water, may be derivatized to become insoluble, e.g. by cross-linking and/or by coupling to an insoluble body via adsorption or covalent binding. Hydrophilic groups can be introduced on hydrophobic polymers (e.g. on copolymers of monovinyl and divinylbenzenes) by polymerization of monomers exhibiting groups which can be converted to OH, or by hydrophilization of the final polymer, e.g. by adsorption of suitable compounds, such as hydrophilic polymers.

The base matrix can also be based on inorganic material, such as silica, zirconium oxide, graphite, tantalum oxide etc. Preferred matrices lack hydrolytically unstable groups, such as silan, ester, amide groups and groups present in silica as such. This is in particular important when recycling and reusing the matrix for removal of the plasmid from subsequent batches of samples.

The matrix may be porous or non-porous. This means that the matrix may be fully or partially permeable (porous) or completely impermeable to the plasmid to be removed (non-porous).

In a particularly interesting embodiment of the present invention, the bed may comprise a population of particles that may be irregular or preferably spherical (beads). The mean size of the particles of the population is typical in the interval 10-500 μm, such as ≥ 50 μm. The preference is for mean sizes ≥ 100 μm or even ≥ 150 μm.

The particles may contain density-controlling filler particles. This may be benificial in process types in which there are steps utilizing expanded beds and/or settling of the particles. See for instance WO 9218237 (Amersham Pharmacia Biotech AB); WO 9717132 (Amersham Pharmacia Biotech AB); WO 9833572 (Amersham Pharmacia Biotech AB); and WO WO 9200799 (Kem-En-Tek; Upfront Chromatography).

The bed may be a packed bed, an expanded bed or a turbulent suspension (batch procedure). In at least the packed bed and the fluidised bed there is a liquid flow between the particles and going from one side of the bed to another.

The base matrix may also be a porous monolithic plug or membrane having pores permitting the liquid flow to pass through.

In a preferred embodiment of the present invention the anion exchange groups are bound to the base matrix via extenders on inner surfaces (pore surfaces) and/or outer surfaces of the matrix. See for instance WO 9833572 (Amersham Pharmacia Biotech AB) the content of which hereby is enclosed by reference. Another commonly used word for extenders is tentacles. The benefit of extenders is that they are likely to cause an increase in the effective interaction volume as well as in the steric availability of the weak anion exchange groups. This in turn will increase the mass transfer rate as well as the total capacity.

Suitable extenders should be hydrophilic and contain a plurality of groups selected from e.g. hydroxy, carboxy, amino, repetitive ethylene oxide ($-CH_2CH_2O-$), amido etc. The extender may be in the form of a polymer. Hydrophilic polymeric

extenders may be of synthetic origin or of biological origin. Typical synthetic polymers are polyvinyl alcohols, polyacryl- and polymethacrylamides, polyvinyl ethers etc. Typical biopolymers are polysaccharides, such as starch, cellulose, dextran, agarose. The preferred polymeric extenders are often water-soluble in their free state, i.e. when they are not present in the base matrix.

The length (size) of the optimal extender will depend on several factors, such as number of attachment points to the base matrix of the beads, type of extender, type and size of anion groups etc. For polymeric extenders for which attachment and/or cross-linking is possible at several monomeric units, it is believed that larger extenders are preferred. It is believed that the most suitable polymers should contain at least 30 monomeric units, which for polysaccharides like dextran indicates a M_w > 5000 Da.

The spacer SP may be a straight, branched or cyclic divalent hydrocarbon group linking the ligand to the matrix. The spacer may have a C_{1-20} hydrocarbon chain that may be (a) broken at one or more locations by an amino group -NHR^4- and/or ether oxygen (-O-) and/or thioether sulphur (-S-) and/or (b) substituted with one or more amino groups (-NR^5,R^6) and/or one or more -OR^7 groups. In case the weak anion exchange ligand is a primary, seondary or tertiary ammonium group there may be a hydroxy group at 2 or three atoms distance from the nitrogen atom of such an ammonium group. The hydrocarbon group may contain saturated, or unsaturated or aromatic structures.

The ligand is in the preferred case a group ($R^1R^2R^3$)N in protonated form, i.e. a primary, secondary or tertiary ammonium group. SP binds to L by replacing one of R^1, R^2 , and R^3.

Above R^{1-7} represent groups selected among hydrogen or a straight, branched or cyclic C_{1-10} hydrocarbon groups that may contain saturated, unsaturated and/or aromatic structures. The hydrocarbon groups may have

(a) a C_{1-10} hydrocarbon chain that may be broken at one or more locations by an amino group $-NHR^8-$ and/or an ether oxygen (-O-) and/or a thioether sulphur (-S-) and/or

(b) being substituted with one or more amino groups ($-NR^9R^{10}$) and/or one or more $-OR^{11}$.

R^{8-11} are typically selected from C_{1-3} alkyls in which one or more of the hydrogens may be replaced with an hydroxy or an amino group.

In both the ligand and the spacer sp^3-hybridised carbon atoms typically binds to at most one oxygen or nitrogen atom in addition to carbon and hydrogen atoms.

The number of ligands in a suitable anion exchange matrix depends on the particular matrix used, presence and type of extenders, type of spacer and the particular ligand. Based on present knowledge the optimal amount has to be determined from case to case. It is however believed that the number of ligand measured as total chloride ion capacity always should be above 50 µmol/ml wet matrix. It has been found that the number of ligands should be above 150 µmol/ml wet matrix for particulate matrices having the following features: (a) mean particle size above 100 µm, (b) dextran extenders, and (c) diethylamino groups as ligands preferentially bound to an extender via a spacer ending in $-CH_2CHOHCH_2-$.

The best mode obtained at the filing date is presented in the experimental part.

The invention is further defined in the appended claims. The invention is illustrated by the non-limiting examples given in the Experimental Part.

EXPERIMENTAL PART

SECTION A: SYNTHESIS OF ANION EXCHANGERS

EXAMPLE 1: Preparation of strong anion exchange adsorbent (Q)

The strong anion exchange adsorbent was prepared according to the method described in WO 9833572 (Amersham Pharmacia Biotech AB), Example 6. The amount of G-MAC (glycidyltrimethyl ammonium chloride) was adapted to give a product withchloride ion capacity that was was 0.28 mmol/ml adsorbent. The mean size (diameter) of the beads produced was 200 µm.

EXAMPLE 2: Preparation of weak anion exchange adsorbent (ANX).

Allylation was performed according to the method described in WO 9833572 (Amersham Pharmacia Biotech AB), Example 7. Analysis showed 0.19 mmol allyl groups per ml adsorbent.

To 1500 ml of allyl adsorbent and 380 ml distilled water in a reactor were added under stirring 60 g of sodium acetate and 30 ml bromine. After 5 minutes of reaction time a solution of sodium formate was added until all excess of bromine was destroyed. Diethylamine 600 ml was added and pH was adjusted to 11.5, after which the reaction was stirred for 16 hours at room temperature.

The mixture was then neutralised with acetic acid (pH 6-7) and the matrix washed with distilled water.

The chloride ion capacity of the product was 0.16 mmol chloride ions/ml adsorbent. The mean size (diameter) of the beads produced was 200 µm.

METHODS OF ANALYSIS

Allyl contents of various gels were determined on a Mettler DL40GP Memo Titrator with 0.1 M $AgNO_3$.

Chloride ion capacity was determined on a Mettler DL40GP Memo Titrator with 0.1 M $AgNO_3$.

SECTION B: PLASMID PREPARATION

Basis for sample preparation is the classical alkaline lysis protocol (Birnboim et al., Nucleic Acids Res. 7 (1979) 1513-1523). See also Horn et al., Human Gene Therapy 6 (1995) 565-573; and Birnboim, Meth. Enzymol. 100 (1983) 243-255).

Starting from frozen cell pellets (per 50 g of E.coli cells harbouring a 4555 bp plasmid) the following steps were carried out:

- Add 390 ml of suspension buffer (61 mM in glucose, 10 mM Tris, 50 mM EDTA, pH 8, kept on ice), allow the cells to get in suspension on ice, (stirring at low speed <200rpm, turbine stirrer, ratio of stirrer diameter to vessel diameter between 0.5 to 0.6).
- Add 780 ml of lysis buffer (0.2 N NaOH, 1% in SDS). Lysis buffer was kept at RT before addition to the suspension mix kept on ice (10 mins stirring at <200rpm).
- Add 585 ml of KOAc solution (important: 295 g KOAc, 115 ml of acetic acid filled up with water to 1L, no pH adjustment. Just for reference: 135 mS/cm, pH 5.6). 10 mins stirring at < 200 rpm.
- Add 195 ml of ice cold 2 M $CaCl_2$ solution. Stirring at <200 rpm for 10 mins on ice.
- Allow to precipitate overnight at 4°C (or corresponding time during day-time)
- Centrifuge at 9000 rpm (14000xg) for 20 mins.
- Supernatant is filtered through 0.45 μm filter.
- volume of filtrate is reduced by ultrafiltration (UF) using Ultrasette (Filtron/Pall, catalog number: OS100C70; polyether sulfone, 100,000 MWCO Pore Size, screen channel, 836 cm^2 filter area, Filtron, Northborough, MA, U.S.A.) from about 1.8 L to 100ml. UF unit is rinsed with 100 ml of water, resulting in about 200ml. In order to achieve a conductivity to be below 50 mS/cm the solution has to be diluted with water. The final volume is supplemented with EDTA (to an excess of 5 mM over the $CaCl_2$ conc present before UF) and Triton X-100 (to 0.02 %) before application to the capture step.

CAPTURE STEP

System: ÄKTA™explorer (Amersham Pharamacia Biotech, Uppsala, Sweden).

Column: XK 16/15 (30 ml gel) packed with ANX anion exchanger.

Flow velocity: 150 cm/h.

Sample origin: 22 g cells.

Sample volume: 480 ml (approx. 16 CV).

Washing: Buffer A (4 CV).

Elution: Gradient 0-30% Buffer B in 3.3 CV.

Buffer A: 30 mM KOAc, 0.5 M NaCl, 5 mM EDTA, 0.02 % Triton X100, pH 5.1 (50 mS/cm at 25°C).

Buffer B: 30 mM KOAc, 1 M NaCl, 5 mM EDTA, pH 5.1.

Elution profile: Unbound material eluted after one CV of buffer A (0 % B). Bound material eluted within 3 CV (at 56 mS/cm) with a linear gradient up to 30% B. Subsequently a step gradient to 100 % B was applied.

Analysis: The method was agarose gel (1 %) electrophoresis, ethidium bromide staining and visualisation by UV. No detectable amounts of plasmid could be seen in the flow through fraction when loading up to 0.5 cell equivalents per ml gel. RNA could be detected in the bound fraction and was estimated to be less than 5 %.

Recovery: For the capture step this was calculated to > 95 %.

Comparison between the Q and ANX anion exchangers.

System: ÄKTA™explorer 100 (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Column: PEEK column (4.6x10) (1.66 ml gel), packed with the ANX anion exchanger or the Q-anion exchanger adsorbents prepared as described above.

Flow velocity: 108 cm/h

Sample volume: 5 ml (approx. 3 CV), originally deriving from 0.6 g of cells per scouting run.

Washing: Buffer A (3 CV).

Elution: Gradient 0-100 % Buffer B in 10 CV.

Buffer A	30 mM potassium acetate, 0.5 M NaCl, pH 5.1 (corrected to 50 mS/cm at 25°C).
Buffer B	30 mM potassium acetate, 1.0 M NaCl, pH 5.1.

Fractions corresponding to the flow through volume and the bound fraction eluting in the gradient were in each experiment analysed by ethidium bromide stained agarose (1 %) gel electrophoresis. In both experiments the flow through fraction contained RNA together with small amounts of plasmid. For the Q anion exchanger the bound fraction eluted as a small double peak at a slightly higher conductivity compared to the ANX anion exchanger. For the Q anion exchanger the gel electrophoresis results suggested a very low recovery of plasmid compared to the ANX anion exchanger under the same conditions.

C L A I M S

1. A method for purifying plasmid on a bed composed of an anion exchange matrix bed comprising a plurality of anion exchange groups firmly attached to a hydrophilic base matrix, **characterized** in that

   A. essentially all of the anion exchanging ligands are weak anion exchange ligands, and

   B. the method comprises the steps:

      (a) providing a plasmid sample derived from a cleared lysate of recombinant host cells producing the plasmid;

      (b) contacting said sample with the bed of the anion exchange matrix, said contacting being under conditions enabling binding of said plasmid to said anion exchange matrix;

      (c) desorbing selectively said plasmid from said anion exchange matrix, and

      (d) if so desired, regenerating the matrix after an optional cleaning step, and repeating step (b) with another batch of sample provided according to step (a)

   steps b-c and step d, if present, preferably being carried out in one and the same vessel.

2. The method according to claim 1, **characterized** in (a) that plasmid sample derives from cells lysed by an alkaline lysis method.

3. The method according to anyone of claims 1-2, **charaterized** in that the plasmid sample has a level of chelator that is in excess for chelating divalent metal ions in the sample.

4. The method according to anyone of claims 1-3, **characterized** in that the detergent concentration in the plasmid sample is in the interval 0.005-0.2% (w/w).

5. The method according to anyone of claims 1-4, **characterized** in that the plasmid sample derives from a cell lysate which has been treated to reduce its content of ribonucleic acid, for instance by precipitation and/or size exclusion filtration, such as ultra filtration or size exclusion chromatography.

6. The method according to anyone of claims 1-5, **characterized** in that the conductivity of the sample applied to the anion exchanger is at least 40% but below 99 %, preferably 5-20 %, below the elution conductivity for the plasmid.

7. The method according to anyone of claims 1-6, **characterized** in that the plasmid sample derives from a cell lysate and that the volume has been reduced by ultrafiltration through a membrane with for instance a molecular size cut off value in the interval 75,000-150,000 KD for plasmids in the interval 2-6 kbp.

8. The method according to anyone of claims 1-7, **characterized** in that the bed is composed by a population of particles having a mean size in the range 10-500 μm preferably ≥ 50 μm, more preferably ≥ 150. μm.

9. The method according to anyone of claims 1-8, **characterized** in that the matrix is porous.

10. The method according to anyone of claims 1-9, **characterized** in that the matrix is in form of a porous bed, that a liquid flow is applied through the bed and that the liquid flow velocity through the matrix is adapted to the bed height such that each part volume of the sample will have a residence time in the interval 1-30 min.

11. The method of anyone of claims 1-10, **characterized** in that the weak anion exchanging ligands are selected among primary, secondary or tertiary ammonium groups.

12. The method according to anyone of claims 1-11, **characterized** in that

(a) the weak anion exchanging groups are ammonium groups that are linked via a spacer to the base matrix, and

(b) the spacer has a hydroxy group at a distance from 2-3 atoms distance from the nitrogen atom in the amino group,

with preference for said 2-3 atoms are carbon atoms.

13. The method of anyone of claims 1-12, **characterized** in the base matrix comprises extenders carrying one or more spacers with a weak anion exchange ligand.

14. The method according to anyone of claims 1-13, **characterized** in that the base matrix with attached anion exhange ligands comprises the structure

$$[(R^1R^2R^3)N]\text{-----}SP\text{-}M$$

in which

- ----- represents that SP is binding to the ligand $(R^1R^2R^3)N$ by replacing one of R^1, R^2 , and R^3;
- SP represents a spacer having a straight, branched or cyclic divalent hydrocarbon group linking the ligand to the matrix and having a C_{1-20} hydrocarbon chain that may be broken at one or more locations by an amino group -NHR^4- and/or ether oxygen (-O-) and/or thioether sulphur (-S-) and/or being substituted with one or more amino groups (-NR^5R^6) and/or one or more -OR^7 groups;
- R^{1-7} represent groups selected hydrogen or a straight, branched or cyclic C_{1-10} hydrocarbon groups having
  (a) a C_{1-10} hydrocarbon chain that may be broken at one or more locations by an amino group -NHR^8- and/or an

ether oxygen (-O-) and/or a thioether sulphur (-S-) and/or

(b) being substituted with one or more amino groups (-NR^9R^{10}) and/or one or more -OR^{11},

with R^{8-11} being selected from C_{1-3} alkyls in which one or more of the hydrogens may be replaced with a hydroxy or an amino group.

15. The method of anyone of claims 1-14, **characterized** in that

- the anion exchange matrix comprises
  - (I) a particulate base matrix having (a) a mean particle size above 100 µm, and (b) dextran extenders, and
  - (II) diethylamino groups as ligands preferentially bound to the extenders via spacer ending in -$CH_2CHOHCH_2$-; and
- a total chloride ion capacity of the anion exchange matrix is ≥ 150 meq/ml wet matrix.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/04433

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 B01J41/00 B01J41/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N B01J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, CHEM ABS Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 29825 A (PHARMACIA BIOTECH AB ;BERGLUND ROLF (SE); BERGSTROEM JAN (SE); BEL) 21 August 1997 (1997-08-21) page 6 -page 10; claims	1,9,12, 14,15
X	WO 99 16869 A (AMERSHAM PHARM BIOTECH AB ;BHIKHABHAI RAMAGAURI (SE)) 8 April 1999 (1999-04-08) page 5 -page 10; claims	1-6,9, 10,13
Y		8
Y	WO 98 33572 A (AMERSHAM PHARM BIOTECH AB ;BERG HANS (SE); HANSSON HASSE (SE); KAA) 6 August 1998 (1998-08-06) page 8 -page 10	8

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040. Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Armandola, E

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/04433

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9729825 A	21-08-1997	AU 713635 B	09-12-1999
		AU 1818097 A	02-09-1997
		CA 2242927 A	21-08-1997
		EP 0888157 A	07-01-1999
		JP 2000504625 T	18-04-2000
		US 6090288 A	18-07-2000
WO 9916869 A	08-04-1999	EP 0964923 A	22-12-1999
WO 9833572 A	06-08-1998	AU 6009898 A	25-08-1998
		EP 1003599 A	31-05-2000